THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 266, No. 15, Issue of May 25, pp. 9971-9976, 1991
Printed in U.S.A.

The Role of Cysteines in Polyketide Synthases

SITE-DIRECTED MUTAGENESIS OF RESVERATROL AND CHALCONE SYNTHASES, TWO KEY ENZYMES IN DIFFERENT PLANT-SPECIFIC PATHWAYS*

(Received for publication, December 28, 1990)

Thomas Lanz, Susanne Tropf, Franz-Josef Marner‡, Joachim Schröder, and Gudrun Schröder

From the Institut für Biologie II, Lehrstuhl für Biochemie der Pflanzen, Universität Freiburg, Schänzlestrasse 1, D-7800 Freiburg and the ‡Institut für Biochemie, Universität zu Köln, Zülpicherstrasse 47, D-5000 Köln 1, Federal Republic of Germany

Resveratrol and chalcone synthases are related plant-specific polyketide synthases that are key enzymes in the biosynthesis of stilbenes and flavonoids, respectively. The stepwise condensing reactions correspond to those in other polyketide and fatty-acid synthases. This predicts that the two proteins also contain cysteines that are essential for enzyme activity because they bind the substrates. We exchanged, in both enzymes, all of the 6 conserved cysteines into alanine by site-directed mutagenesis and tested the mutants after expression of the proteins in the *Escherichia coli* heterologous system. Only cysteine 169 was essential in both enzymes, and inhibitor studies suggest that it is the main target of cerulenin, an antibiotic reacting with the cysteine in the active center of condensing enzymes. Most of the other exchanges led to reduced activities. In two cases, the enzymes responded differently, suggesting that the cysteines at positions 135 and 195 may be involved in the different product specificity of the two enzymes. The sequences surrounding the essential cysteine 169 revealed no similarity to the active sites of condensing enzymes in other polyketide synthases and in fatty acid biosynthesis. The available data indicate that resveratrol and chalcone synthases represent a group of enzymes that evolved independently of other condensing enzymes.

Chalcone synthase (Fig. 1, *CHS*) is a plant-specific enzyme that synthesizes naringenin chalcone, an essential precursor of flavonoids and of a large number of isoflavonoid phytoalexins. The enzyme performs a condensation of a 4-coumaroyl residue with three two-carbon units from malonyl-CoA, followed by formation of a new aromatic ring system (Kreuzaler and Hahlbrock, 1975a, 1975b). Resveratrol synthase (Fig. 1, *RS*) synthesizes the backbone of stilbene phytoalexins. Resveratrol and chalcone synthases are closely related. Both use the same substrates with the same stoichiometry, and the available data (Schöppner and Kindl, 1984; Kindl, 1985) indicate that the two enzymes use the same condensation mechanism. The protein sequences reveal similarities of ~70% between resveratrol and chalcone synthases from different sources. It seems likely that the formation of different products is due to a different spatial stabilization of tetraketide intermediates directly prior to formation of the ring systems (Schröder and Schröder, 1990).

Several properties of chalcone synthase support a model that the condensing reactions are very similar to those in other polyketide synthases and in fatty acid biosynthesis: (i) stepwise elongation of a starter group by addition of acetate units from malonyl groups (Hrazdina *et al.*, 1976; Schüz *et al.*, 1983); (ii) sensitivity to inhibitors reacting with cysteine -SH groups, for example, cerulenin and iodoacetamide (Kreuzaler and Hahlbrock, 1975b); (iii) two side reactions also found with fatty-acid synthase (malonyl-CoA decarboxylation and CO_2 exchange) (Kreuzaler *et al.*, 1978); and (iv) acceptance of butyryl-CoA and hexanoyl-CoA as starter molecules (Schüz *et al.*, 1983).

The starter molecule for the elongation step in fatty acid biosynthesis is covalently bound to the -SH group in an enzyme cysteine prior to the condensation, and the malonyl residues are attached to the 4′-phosphopantetheine -SH group in acyl carrier protein (see Wakil (1989) for review). The cysteine -SH group in the condensing enzyme is the target of the inhibitors iodoacetamide and cerulenin (Kresze *et al.*, 1977; Kauppinen *et al.*, 1988; Funabashi *et al.*, 1989).

Based on the similarities to the condensing steps in fatty acid synthesis, the first model of the chalcone synthase reaction suggested covalent binding of both substrates to the enzyme (Kreuzaler and Hahlbrock, 1975b). Later experiments showed that chalcone synthase does not possess the 4′-phosphopantetheinyl residues characteristic for acyl carrier protein function. Since purified chalcone synthase requires no additional factors for activity (Kreuzaler *et al.*, 1979), it remained unanswered whether an -SH group in the enzyme serves as acyl carrier protein equivalent or whether malonyl-CoA is used directly as substrate.

Taken together, these comparisons suggest the following predictions. (*a*) Chalcone and resveratrol synthases should possess a cysteine for covalent binding of the starter 4-coumaroyl residue. This amino acid should be essential for enzyme activity, and its sulfhydryl group should be the target of cerulenin (Omura, 1981). The active sites in several condensing enzymes have been identified either by biochemical experiments or by sequence comparisons. The alignment in Fig. 2*A* indicates that the cysteines are surrounded predominantly by serine/threonine and small neutral amino acids (glycine or alanine). A search for related motifs in resveratrol and chalcone synthases suggested 1 or 2 cysteines as candidates for the active site (Fig. 2*B*) (Kauppinen *et al.*, 1988; Schröder and Schröder, 1990). (*b*) The two enzymes may contain a second essential sulfhydryl group, if binding of the malonyl residue to the enzymes is essential for activity.

We tested these predictions by site-directed mutagenesis of all cysteines that are strictly conserved in both resveratrol and chalcone synthases, and we investigated the activity and

* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 206 and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

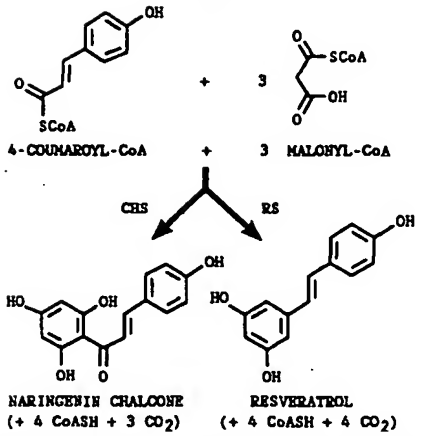

FIG. 1. **Reactions performed by chalcone (*CHS*) and resveratrol (RS) synthases.**

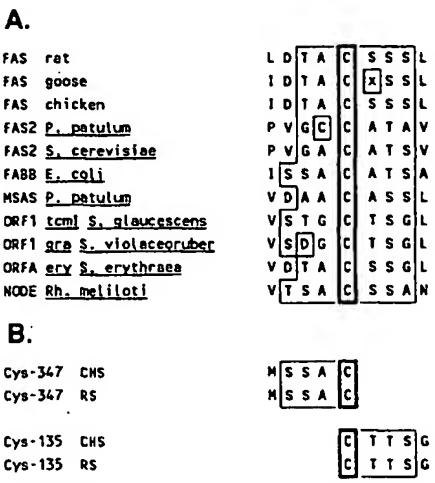

FIG. 2. *A*, active sites in condensing proteins of fatty-acid (*FAS*) and polyketide synthases. The sequences are from the following references: *FAS rat*, Schweizer *et al.* (1988); *FAS goose* (*X*, unidentified amino acid), Poulose *et al.* (1984); *FAS chicken*, Chang and Hammes (1988); *FAS2 P. patulum* (fatty-acid synthase subunit 2 from *Penicillium patulum*), Wiesner *et al.*, 1988; *FAS2 S. cerevisiae* (fatty-acid synthase subunit 2 from *Saccharomyces cerevisiae*), Schweizer *et al.*, 1987; *FABB E. coli* (2-ketoacylsynthase B from *E. coli*), Kauppinen *et al.*, 1988; *MSAS P. patulum* (methylsalicylic-acid synthase from *P. patulum*), Beck *et al.*, 1990; *ORF1 tcmI S. glaucescens* (open reading frame 1 in the *tmcI* locus of *Streptomyces glaucescens*), Bibb *et al.*, 1989; *ORF1 S. violaceoruber* (open reading frame 1 in the *gra* locus of *Streptomyces violaceoruber*) Sherman *et al.*, 1989; *ORFA ery S. erythraea* (erythromycin-producing polyketide synthase from *Saccharopolyspora erythraea*), Cortes *et al.*, 1990; *NODE Rh. meliloti* (protein encoded in gene *nodE* of *Rhizobium melioti*), Debelle and Sharma, 1986. *B*, active-site cysteines proposed for chalcone (*CHS*) and resveratrol (RS) synthases (Kauppinen *et al.*, 1988; Schröder and Schröder, 1990).

cerulenin inhibition of the unchanged and mutant proteins after expression in the *Escherichia coli* heterologous system.

MATERIALS AND METHODS

Vectors, Helper Phage, Bacterial Strains, and Standard Cloning Procedures—The following vectors and phage were used: pTZ18R and pTZ19R (Zagursky and Berman, 1984), pINIIA3 (Nakamura and Inouyé, 1982), and helper phage M13K07 (Vieira and Messing, 1987). The cloning experiments were performed in *E. coli* strain JM109 (Yanisch-Perron *et al.*, 1985) following standard procedures (Sambrook *et al.*, 1989).

Modification of pTZ19R—The plasmid was digested with *Eco*RI and *Sma*I and religated after fill-in of protruding ends with the Klenow fragment of DNA polymerase I. The *Sph*I site was then removed by religation of *Sph*I-digested plasmid treated with S1 nuclease. The result is a modified pTZ19R vector with a disruption in the β-galactosidase coding region. The plasmid retains the sites for *Bam*HI, *Xba*I, *Sal*I, *Pst*I, and *Hin*dIII in the polylinker region.

pRS3: Construction of Resveratrol Synthase Clone for Functional Enzyme Expression in E. coli—RS3 has been described (Lanz *et al.*, 1990). It is a 2.7-kilobase pair genomic *Eco*RI fragment from *Arachis hypogaea* that, except for a few base pairs at the amino-terminal end, contains the complete protein coding region, which is split by a single intron.

The reconstruction for protein expression in *E. coli* is summarized in Fig. 3*A*. The fragment was first shortened by deletion of a 0.4-kilobase pairs *Eco*RV/*Sma*I fragment in the 3'-noncoding region; the *Sma*I site was from the polylinker of vector pTZ19R. The intron was removed by deletion mutagenesis with an oligonucleotide that joined the two exons. The fragment was then recloned with *Eco*RI/*Bam*HI into vector pINIIA3 digested with the same enzymes. This fused the three amino-terminal amino acids of the *E. coli* prolipoprotein (Nakamura and Inouyé, 1982) in-frame to the open reading frame of RS3, and it also supplied a bacterial ribosome-binding site from the vector (in the small *Xba*I/*Eco*RI fragment). The combination was excised with *Xba*I/*Bam*HI and recloned into modified vector pTZ19R digested with the same enzymes. This provided an inducible promoter for expression of the protein; the use of a modified vector was necessary to avoid a translational fusion with the β-galactosidase gene from the vector (Fig. 3*B*). The sequences were verified by sequencing.

pCHS: Construction of Chalcone Synthase Clone for Functional Enzyme Expression in E. coli—cDNA clone pSCHS3 from *Sinapis alba* has been published (Ehmann and Schäfer, 1988); it contains the complete coding region. The *Eco*RI cDNA fragment was recloned into the *Eco*RI site of vector pTZ18R to provide an inducible promoter for the expression of the protein. In this case, a modification of the vector was not necessary because β-galactosidase and chalcone synthase use different reading frames. The sequences were verified by sequencing.

Site-directed Mutagenesis—The oligonucleotides are shown in Fig. 3*A* and Table I. They were synthesized with a DNA synthesizer from

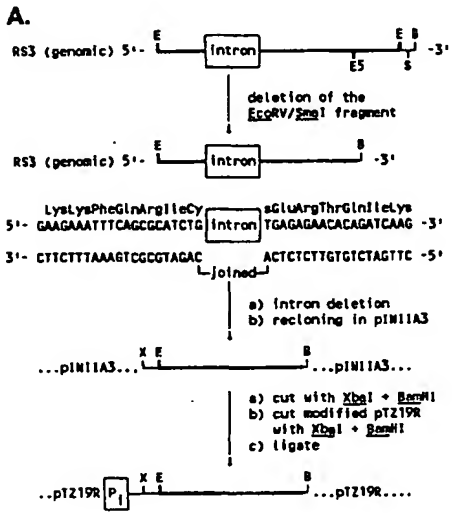

FIG. 3. **Construction of resveratrol synthase clone pRS3 for inducible protein expression in *E. coli* (see "Materials and Methods" for description).** *A*, cloning steps; *B*, details of the protein start region. The distances are not drawn to scale, and only the sites used for cloning are shown. The *thick black lines* indicate resveratrol synthase or chalcone synthase sequences; the *thin black lines* indicate vector sequences. *B*, *Bam*HI; *E*, *Eco*RI; *E5*, *Eco*RV; *S*, *Sma*I; *X*, *Xba*I; P_I, inducible promoter of the pTZ19R vector; ***, stop codon.

TABLE I

Oligonucleotides for the change of cysteine into alanine

The exchanged nucleotides are underlined.

Cys	Resveratrol synthase
65	5'-GCGCATC-GCT-GAGAGA
89	5'-CCTAACATG-GCT-GCATAC
135	5'-TTTGATCTTC-GCC-ACCACC
169	5'-CCAAGGT-GCC-TTCGCT
195	5'-CTTATCGTT-GCT-TCTGAG
347	5'-GTCAAGTGCC-GCT-GTGTTC
Cys	**Chalcone synthase**
65	5'-GCGCATG-GCC-GACAAGTC
89	5'-CCAAACATG-GCT-GCCTACATG
135	5'-GTTGTCTTC-GCT-ACTACC
169	5'-CCAGCAAGGT-GCC-TTCGCCG
195	5'-CTCGTAGTC-GCC-TCTGAGATC
347	5'-GAGCGCG-GCT-GTTCTG

Applied Biosystems, Inc. and purified by high-performance liquid chromatography (Birsner *et al.*, 1987). For mutagenesis, the recombinant plasmids were transformed into *E. coli* strain RZ1032 (HfrKL16 PO/45 (*lysA*(61–62)), *dut1*, *ung1*, *thi1*, *relA1*, Zbd-279::Tn*10*, *supE44*), and single-stranded DNA produced in presence of helper phage M13K07 was used for mutagenesis. The strain and the procedures have been described (Kunkel *et al.*, 1987). The experimental protocol was modified in one step: the double-stranded DNA product was methylated with *S*-adenosylmethionine prior to transformation into strain JM109 (Horton and Lord, 1986). The presence of the mutations was verified by DNA sequence analysis. We also confirmed, by several sequential transformation steps, that the *E. coli* cells contained no other than the mutant plasmids.

DNA Sequence Analysis—The sequences were analyzed by the dideoxynucleotide chain termination technique (Sanger *et al.*, 1977; Sanger and Coulson, 1978). The pTZ18R and pTZ19R system, helper phage M13K07, and *E. coli* strain JM109 (all from Pharmacia LKB Biotechnology Inc.) and the reverse sequencing primer (Boehringer Mannheim) or synthetic oligonucleotides were used routinely. DNA polymerization reactions were performed with [^{35}S]dATPαS[1] (≥37 TBq/mmol, Amersham Corp.) and modified T7 DNA polymerase (Sequenase, United States Biochemical Corp.). Sequence compilation and evaluation were carried out with standard programs (Kroeger and Kroeger-Block, 1984). The search of the protein data base (SwissProt Version 14) was performed with program MATSCAN and the Pcgene software package (IntelliGenetics Inc., Mountain View, CA).

Protein Expression in E. coli and Enzyme Extracts—The bacteria containing pRS3 or pCHS were grown to A_{600} = 0.8–1, induced for 3–5 h with 1 mM isopropyl-β-D-thiogalactoside, harvested in 1-A_{600} portions, and stored at −70 °C. The cells were resuspended in 80 μl of 0.2 M Hepes/KOH (pH 7.0) containing 1 mM EDTA, mixed with 10 μl of 1 mg/ml lysozyme dissolved in the same buffer, and lysed for 30 min on ice. After centrifugation in the cold for 10 min at 15,000 × *g*, the supernatant fluids were used for the enzyme assays and Western blots.

Enzyme Assays—The assay conditions were based on previous work (Schröder *et al.*, 1979a), but were adapted to bacterial extracts. The same conditions were found to be optimal for resveratrol and chalcone synthases. The assays described below satisfied the requirement that product formation was linear with time and protein concentration. The incubations contained 15 μl of enzyme extract, 75 μl of buffer (0.2 M Hepes/KOH (pH 7.0), 1 mM EDTA), 5 μl of 4-coumaroyl-CoA (1 nmol), and 5 μl of [2-^{14}C]malonyl-CoA (1.5 nmol, 0.78 GBq/mmol, Amersham Corp.). Assays with cerulenin ((2*R*,3*S*)-2,3-epoxy-4-oxo-7,10-*trans*,*trans*-dodecadienamide) were preincubated with the antibiotic for 10 min at 25 °C prior to start of the reaction by addition of the substrates (Kreuzaler and Hahlbrock, 1975b). Ethylene glycol monomethyl ether was used as solvent for cerulenin, and <4 μl was added to the assays; the solvent had no effect on the enzyme reactions in final concentrations up to 4%. After 10–20 min at 37 °C, the incubations were stopped by two sequential extractions with 0.15 ml of ethyl acetate. The solvent was removed with a SpeedVac concentrator. The residue was redissolved in 10 μl of ethyl acetate, and 0.5 μg of resveratrol and 0.5 μg of naringenin were added as internal standards. Routine samples were analyzed by thin-layer chromatography (precoated cellulose, E. Merck AG) in 15% acetic acid. The radioactive products were quantified with an automatically integrating TLC linear analyzer (LB2842, Berthold, Wildbad, Federal Republic of Germany).

Specific enzyme activities were not based on total protein because the preparation of the extracts included addition of lysozyme and because the rate of protein expression varied between the mutants. Therefore, the following procedure was applied for each series of experiments. Each extract used for the enzyme measurements was also analyzed by quantitative Western blotting for resveratrol or chalcone synthase protein. Quantification was performed with a laser densitometer (Ultroscan, LKB, Bromma, Sweden). This procedure allowed a definition of enzyme activities that is based on the quantification of the specific proteins in the incubations rather than on total protein in the *E. coli* extracts.

Antisera and Western Blots—The antiserum against chalcone synthase has been published (Schröder *et al.*, 1979b). The resveratrol synthase antiserum was obtained against an overexpressed bacterial fusion protein. The DNA region encompassing 195 amino acids from the carboxyl-terminal end of resveratrol synthase (position 200 to end in Fig. 6) was fused in-frame with the amino-terminal part of MS2 polymerase in expression vector pEX31b. The vector, the expression of the fusion protein and its purification, and the raising of antiserum in rabbits have been described in detail (Strebel *et al.*, 1986). Western blotting was performed as described (Schindler *et al.*, 1989).

Analytical Techniques for Resveratrol Identification—For HPLC, the ethyl acetate-extracted and dried samples were dissolved in methanol and separated with a Kontron Model 200 HPLC apparatus. The column used was 5-μm LiChrospher 100 RP_{18} (125-mm LiChrocart cartridge, 4-mm internal diameter, E. Merck AG). The solvent gradient was 1 ml/min H_2O/methanol (60:40, v/v) to H_2O/methanol (40:60, v/v) within 10 min and then to 100% methanol within 10 min. Detection was with a Hewlett-Packard 1040A diode array detector at 306 nm. A UV spectrum (200–400 nm) was recorded every 0.64 s. Resveratrol was identified by comparison of the retention time and the UV spectrum with the authentic standard.

For gas chromatography-mass spectrometry, after evaporation of the solvent, the samples were dissolved in *N*-methyl-*N*-trimethylsilyltrifluoroacetamide and allowed to react for 10 min at room temperature. A Finnigan MAT 4510 gas chromatography-mass spectrometry apparatus was used for separation and identification. Gas chromatography conditions were as follows: 15-m (0.25-mm internal diameter) WCOT-fused silica column (SE30, CB-phase, Chromatography-Service), 100 °C (1 min), and 10 °C/min to 330 °C. Mass spectra (electron impact, 70 eV) were recorded in full-scan mode (35–650 atomic mass units/s) or, for samples with low concentrations, in multiple ion detection mode (observing *m/z* 73 and 444, 0.4 s each). The retention time and the mass spectrum (or the ratio of ions 73 and 444 in case of multiple ion detection) in comparison with a silylated resveratrol standard were used to confirm the identity of the products.

RESULTS

Heterologous System: Functional Expression of Resveratrol and Chalcone Synthases in E. coli—Fig. 4 shows that plasmid pRS3, which contains the resveratrol synthase construction, expressed in *E. coli* a protein that reacts with the resveratrol synthase antiserum and is of the expected size (43 kDa). Very long exposures of the blots revealed a weak cross-reaction with chalcone synthase. This was expected from a polyclonal antiserum and from the sequence similarities between the two proteins (Fig. 6). Extracts from the *E. coli* cells with pRS3 possessed resveratrol synthase activity (Fig. 5). The identity of the product was confirmed by HPLC and mass spectrometric analysis.

The same expression experiments were performed with plasmid pCHS, which was constructed for chalcone synthase. The results are also summarized in Figs. 4 and 5. The polyclonal antiserum against chalcone synthase cross-reacted with resveratrol synthase, but the two proteins are distinguished

[1] The abbreviations used are: dATPαS, adenosine 5'-*O*-(1-thiotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography.

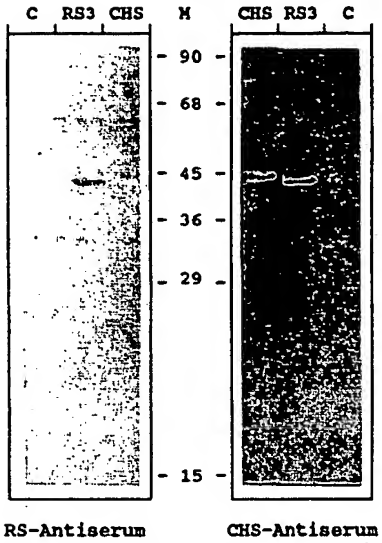

FIG. 4. **Western blot analysis of resveratrol (*RS*) and chalcone (*CHS*) synthases expressed in *E. coli*.** *C*, extracts from control cells without the cloned genes; *M*, marker proteins (in kilodaltons).

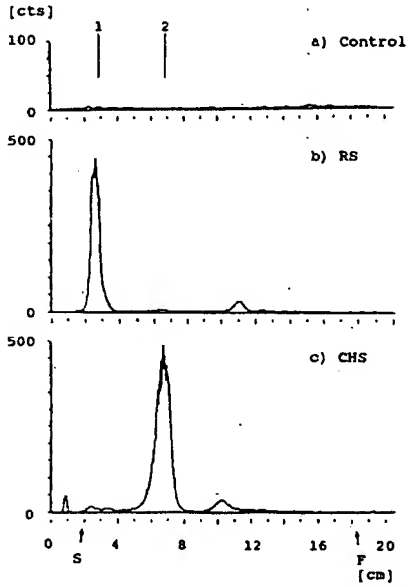

FIG. 5. **Thin-layer chromatographic analysis of radioactive products synthesized with resveratrol (*RS*) and chalcone (*CHS*) synthase proteins in *E. coli* extracts.** *Control*, extracts from cells without the cloned genes. The numbers indicate the positions of authentic reference substances: *1*, resveratrol; *2*, naringenin. *S*, start; *F*, front; *cts*, counts.

by their slightly different sizes (44.5 and 43 kDa; resveratrol synthase is shorter at the amino-terminal end than chalcone synthase) (Fig. 6). The enzyme assays identified naringenin instead of naringenin chalcone as the product of the reaction. This was expected from the nonenzymatic conversion of the chalcone into naringenin, which occurs under all assay conditions (Heller and Hahlbrock, 1980).

The results show that the plant-specific enzymes resveratrol and chalcone synthases are active when expressed in *E. coli*. The use of this heterologous system with the genes cloned in the pTZ vectors has two advantages. (i) The pTZ plasmids can be obtained as single-stranded DNA suitable for the mutagenesis; and (ii) *E. coli* has no genes for these proteins.

```
RS3       MVSVSGIRKVQRAEGPATVLAIGTANPPNCIDQSTYADYYFRVTNSEHMTDLKKK
CHS   MVMGTPSSLDEIRKAQRADGPAGILAIGTANPANHVIQAEYPDYYFRITNSEHMTDLKEK

          65                  89
RS3   FQRICERTQIKNRHMYLTEEILKENPNMCAYKAPSLDAREDMMIREVPRVGKEAATKAIK
CHS   FKRMCDKSTIRKRHMHLTEEFLKDNPNMCAYMAPSLDARQDIVVVEVPKLGKEAAVKAIK

                  135                 169
RS3   EWGQPMSKITHLIFCTTSGVALPGVDYELIVLLGLDPCVKRYMMYHQGCFAGGTVLRLAK
CHS   EWGQPKSKITHVVFCTTSGVDMPGADYQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAK

                  195
RS3   DLAENNKDARVLIVCSENTAVTFRGPSETDMDSLVGQALFADGAAAIIIGSDPVPEV-EK
CHS   DLAENNRGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFSDGAAAIIVGSDPDTSVGEK

RS3   PIFELVSTDQKLVPGSHGAIGGLLREVGLTFYLNKSVPDIISQNINDALNKAFDPLGISD
CHS   PIFEMVSAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLDEAFKPLGISD

                                          347
RS3   YNSIFWIAHPGGRAILDQVEQKVNLKPEKMKATRDVLSNYGNMSSACVFFIMDLMRKRSL
CHS   WNSLFWIAHPGGPAILDQVEKKLGLKAEKMRATRHVLSEYGNMSSACVLFILDEMRRKSK

RS3   EEGLKTTGEGLDWGVLFGFGPGLTIETVVLRSVAI*
CHS   EDGVATTGEGLEWGVLFGFGPGLTVETVVLHSVPV*
```

FIG. 6. **Alignment of amino acid sequences of RS3 from *A. hypogaea* and of chalcone synthase from *S. alba*.** The *black boxes* indicate the 6 cysteines conserved in all chalcone synthases (*CHS*) and in resveratrol synthase; the positions in the sequence are indicated on *top*. The numbering starts with the first amino acid of chalcone synthase. Consensus sequences of chalcone synthase are *underlined* (Schröder and Schröder, 1990). The *dash* at position 238 indicates the amino acid found only in chalcone synthase from *Cruciferae*, but not in other chalcone synthases or in resveratrol synthase.

TABLE II

Enzyme activities of resveratrol and chalcone synthases and of the cysteine → alanine mutants

Values in parentheses represent mean ± S.D.

Enzyme	Enzyme activity	
	RS[a]	CHS
	%	
Original	100 (17)	100 (9)
Cys^{65}	19 (6)	15 (1)
Cys^{89}	78 (19)	71 (3)
Cys^{135}	28 (7)	77 (5)
Cys^{169}	ND	ND
Cys^{195}	36 (18)	121 (17)
Cys^{247}	48 (11)	44 (4)

[a] RS, resveratrol synthase; CHS, chalcone synthase; ND, not detectable.

This avoids interference of the measurements with endogenous enzyme activities.

Essential Cysteines in Resveratrol and Chalcone Synthases—Fig. 6 shows the alignment of resveratrol synthase from *A. hypogaea* with chalcone synthase from *S. alba*. The two enzymes have 6 cysteines in common. These cysteines are also conserved in all other chalcone synthases known so far (Schröder and Schröder, 1990), indicating that they may be of functional importance. With both enzymes, each of these cysteines was changed separately into alanine by site-directed mutagenesis. The mutations were confirmed by DNA sequence analysis, and expression of the protein was confirmed by Western blots with the antisera. All of the experiments were performed with several independent clones of each mutation, and the identity of the reaction product of resveratrol synthase was confirmed by mass spectrometric analysis.

The results of the enzyme activity determinations are shown in Table II. Only the mutation at cysteine 169 abolished enzyme activity completely, and the results were identical for the two enzymes. Since it could not be rigorously excluded that not the removal of the cysteine, but the change into alanine was responsible for the total loss of activity, the

experiments were repeated with resveratrol and chalcone synthase mutants in which cysteine 169 was changed to serine (data not shown). These proteins also failed to show detectable enzyme activity. These experiments defined that both enzymes contain a single cysteine that is essential for resveratrol and chalcone synthase activities.

Interestingly, the change of cysteine to alanine also reduced the activity in most of the other cases. Often the values were comparable for both enzymes (cysteines 65, 89, and 347). In two cases, we observed large differences. Cysteine 195 appears to be important for resveratrol synthase, but not at all for chalcone synthase; and similar effects were observed with cysteine 135. The responses may reflect an aspect of the different product specificities of the two enzymes.

Sensitivity to Cerulenin—Chalcone synthase, like other condensing enzymes (Omura, 1981), is sensitive to cerulenin (Kreuzaler and Hahlbrock, 1975b); but with resveratrol synthase, this had not been investigated. Fig. 7 shows that the enzyme is inhibited by the antibiotic, but with different kinetics. Resveratrol synthase required 3 μg, but chalcone synthase required only 1 μg/assay for 50% inhibition.

These experiments were also performed with each of the resveratrol and chalcone synthase mutant enzymes that possessed enzyme activity, and the results are summarized in Table III. All of the mutant enzymes were sensitive to cerulenin. This would not be expected if any of these 5 cysteines were the target of the antibiotic. By elimination, this supports a proposal that cysteine 169 is the most likely candidate for the cerulenin-binding amino acid.

In several cases, the mutations increased or decreased the sensitivity to cerulenin. The most obvious example is cysteine 135: resveratrol synthase was less sensitive, but chalcone synthase showed increased inhibition. Similar differences between the two enzymes were also obtained with the mutations at cysteine 195. These are the same amino acids that were of different importance for resveratrol and chalcone synthase activities (Table II).

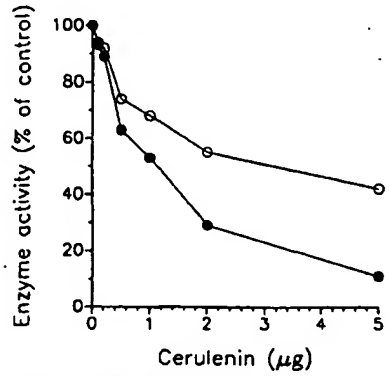

FIG. 7. **Inhibition of resveratrol (○) and chalcone (●) synthases by cerulenin (micrograms/100-μl incubation).**

TABLE III

Activities of resveratrol and chalcone synthases and of the cysteine → alanine mutants in the presence of cerulenin (resveratrol synthase, 3 μg, and chalcone synthase, 1 μg, per assay)

Note that the values are given as percent of the activity measured without the antibiotic.

Enzyme	Enzyme activity	
	RS[a]	CHS
	%	
Original	50 ± 5	50 ± 3
Cys^{65}	52 ± 6	41 ± 10
Cys^{89}	48 ± 3	37 ± 3
Cys^{135}	75 ± 3	27 ± 5
Cys^{195}	49 ± 6	14 ± 1
Cys^{347}	35 ± 3	30 ± 4

[a] RS, resveratrol synthase; CHS, chalcone synthase.

The amino acids surrounding cysteine 169 are highly conserved in chalcone and resveratrol synthases (Fig. 6). The only difference close to cysteine 169 is at position 166, where all known chalcone synthases have glutamine, whereas resveratrol synthase has histidine. Since it seemed possible that this difference contributed to the different sensitivities of chalcone and resveratrol synthases to cerulenin (Fig. 7), we mutated resveratrol synthase histidine 166 to glutamine and chalcone synthase glutamine 166 to histidine.

Table IV shows that with resveratrol synthase, this single change toward chalcone synthases reduced the activity by almost 90%. In contrast to this, with chalcone synthase, the change toward resveratrol synthase led to a reduction by only 30%. The proteins retained their product specificity, *i.e.* they did not possess both enzyme activities. Both of the mutants were cerulenin-sensitive (Table IV), but the rates of inhibition were not significantly affected by the exchanges. The results suggest that the amino acids at position 166 are not major factors in the different inhibitions of resveratrol and chalcone synthases by the antibiotic.

DISCUSSION

Both resveratrol and chalcone synthases possess a single cysteine that is essential for enzyme activity. The similarity of the condensing reaction to those in other polyketide synthases and in fatty-acid synthase (summarized in the Introduction) suggests that this cysteine represents the binding site for the starter 4-coumaroyl group. This proposal is consistent with: (*a*) previous studies of the side reactions malonyl-CoA decarboxylation and CO_2 exchange with chalcone synthase that suggested that the other substrate, the malonyl residue, does not need to be bound to the enzyme to participate in the condensing reaction (Kreuzaler *et al.*, 1978); and (*b*) the indirect evidence that cysteine 169 is the main target for cerulenin that is known to interact specifically with the cysteine of the active site in condensing enzymes. The results support a model that malonyl-CoA serves directly as substrate, and this is different from fatty acid synthesis.

Most of the other cysteine → alanine mutants were reduced in their enzyme activities, suggesting that these changes disturbed some functional aspects of the proteins. Interestingly, resveratrol and chalcone synthases revealed different responses to the changes of cysteines 135 and 195 both in the remaining activity and in the inhibition by cerulenin. The two enzymes utilize the same substrates, but the products are different (Fig. 1). It seems likely that these 2 cysteine residues are involved to some extent in the different product specificity of the enzymes.

TABLE IV

Activities of the mutants at position 166 in the absence and presence of cerulenin (resveratrol synthase, 3 μg, and chalcone synthase, 1 μg, per assay)

Enzyme	Enzyme activity	
	−Cerulenin	+Cerulenin
	%	
RS[a]	100	48 ± 7
RS, His^{166} → Gln	12 ± 1	4 ± 1
CHS	100	46 ± 3
CHS, Gln^{166} → His	70 ± 5	23 ± 1

[a] RS, resveratrol synthase; CHS, chalcone synthase.

Close to cysteine 169, the only difference between the two proteins is at position 166, where all chalcone synthases have glutamine, whereas resveratrol synthase has histidine. The results with the mutants indicate that the difference is of particular importance for resveratrol synthase. The exchanges did not change resveratrol synthase into a chalcone synthase and vice versa, indicating that these amino acids are not the sole factors in determining the product of the reactions.

With the exception of position 166, the environment of the essential cysteine 169 is highly conserved in chalcone and resveratrol synthases (Fig. 6). The sequence in this motif has no similarity to the active site of the condensing enzymes in fatty acid biosynthesis or in polyketide synthases (Fig. 2). A search of protein data bases revealed some similarity to the active site of the condensing enzyme 3-hydroxy-3-methylglutaryl-CoA synthase (Asn-Ala-*Cys*-Tyr-Gly-Gly-Thr-Ala) (Miziorko and Behnke, 1985; Gil *et al.*, 1986), if conservative exchanges are permitted in the comparison. Interestingly, this enzyme is also inhibited by cerulenin (Ohno *et al.*, 1974). It remains to be seen whether these similarities are more than superficial. The protein sequence otherwise has no significant homology to either chalcone or resveratrol synthase.

Based on the similarities of the reaction mechanisms, previous reports on chalcone synthase discussed the possibility that it and the condensing enzymes of fatty acid synthesis have a common evolutionary origin (Schüz *et al.*, 1983). The sequences published in the last few years now permit an evaluation on the level of the protein sequences. The comparison indicates that chalcone and resveratrol synthases have no significant overall similarity to the condensing enzymes in fatty acid or polyketide synthesis, whereas these are related to each other (Hopwood and Sherman, 1990). It still seemed possible, however, that at least the active sites are similar. Our results indicate this is not the case, if the essential cysteine identified in our experiments represents the active site of the condensing reaction. The cysteines suggested by sequence similarity (cysteine 135 or 347) are not essential for enzyme activity and thus do not satisfy the requirement of an active site. Taken together, all of the available data suggest that resveratrol and chalcone synthases represent a closely related group of enzymes that developed independently from the condensing proteins in other polyketide synthases and in fatty acid biosynthesis.

Acknowledgments—We thank E. Schäfer for the chalcone synthase cDNA clone from *S. alba* and E. Schweizer, T. Simpson, and P. von Wettstein-Knowles and her collaborators for stimulating discussions.

REFERENCES

Beck, J., Ripka, S., Siegner, A., Schiltz, E., and Schweizer, E. (1990) *Eur. J. Biochem.* **192**, 487–498

Bibb, M. J., Biro, S., Motamedi, H., Collins, J. F., and Hutchinson, C. R. (1989) *EMBO J.* **8**, 2727–2736

Birsner, U., Gilles, U., Nielsen, P., and McMaster, G. K. (1987) *J. Chromatogr.* **402**, 381–386

Chang, S. I., and Hammes, G. G. (1988) *Biochemistry* **27**, 4753–4760

Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J., and Leadlay, P. F. (1990) *Nature* **348**, 176–178

Debelle, F., and Sharma, S. B. (1986) *Nucleic Acids Res.* **14**, 7453–7472

Ehmann, B., and Schäfer, E. (1988) *Plant Mol. Biol.* **11**, 869–870

Funabashi, H., Kawaguchi, A., Tomoda, H., Omura, S., Okuda, S., and Iwasaki, S. (1989) *J. Biochem. (Tokyo)* **105**, 751–755

Gil, G., Goldstein, J. L., Slaughter, C. A., and Brown, M. S. (1986) *J. Biol. Chem.* **261**, 3710–3716

Heller, W., and Hahlbrock, K. (1980) *Arch. Biochem. Biophys.* **200**, 617–619

Hopwood, D. A., and Sherman, D. H. (1990) *Annu. Rev. Genet.* **24**, 37–66

Horton, R. D., and Lord, S. T. (1986) *Nucleic Acids Res.* **14**, 5112

Hrazdina, G., Kreuzaler, F., Hahlbrock, K., and Grisebach, H. (1976) *Arch. Biochem. Biophys.* **175**, 392–399

Kauppinen, S., Siggaard-Andersen, M., and von Wettstein-Knowles, P. (1988) *Carlsberg Res. Commun.* **53**, 357–370

Kindl, H. (1985) in *Biosynthesis and Biodegradation of Wood Components* (Higuchi, T., ed) pp. 349–377, Academic Press, New York

Kresze, G.-B., Steber, L., Oesterhelt, D., and Lynen, F. (1977) *Eur. J. Biochem.* **79**, 181–190

Kreuzaler, F., and Hahlbrock, K. (1975a) *Arch. Biochem. Biophys.* **169**, 84–90

Kreuzaler, F., and Hahlbrock, K. (1975b) *Eur. J. Biochem.* **56**, 205–213

Kreuzaler, F., Light, R. J., and Hahlbrock, K. (1978) *FEBS Lett.* **94**, 175–178

Kreuzaler, F., Ragg, H., Heller, W., Tesch, R., Witt, I., Hammer, D., and Hahlbrock, K. (1979) *Eur. J. Biochem.* **99**, 89–96

Kroeger, M., and Kroeger-Block, A. (1984) *Nucleic Acids Res.* **12**, 193–201

Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382

Lanz, T., Schröder, G., and Schröder, J. (1990) *Planta (Berl.)* **181**, 169–175

Miziorko, H. M., and Behnke, C. E. (1985) *J. Biol. Chem.* **260**, 13513–13516

Nakamura, K., and Inouyé, M. (1982) *EMBO J.* **1**, 771–775

Ohno, T., Kesado, T., Awaya, J., and Omura, S. (1974) *Biochem. Biophys. Res. Commun.* **57**, 1119–1124

Omura, S. (1981) *Methods Enzymol.* **72**, 520–532

Poulose, A. J., Bonsall, R. F., and Kolattukudy, P. E. (1984) *Arch. Biochem. Biophys.* **230**, 117–128

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanger, F., and Coulson, A. R. (1978) *FEBS Lett.* **87**, 107–110

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467

Schindler, U., Sans, N., and Schröder, J. (1989) *J. Bacteriol.* **171**, 847–854

Schöppner, A., and Kindl, H. (1984) *J. Biol. Chem.* **259**, 6806–6811

Schröder, J., and Schröder, G. (1990) *Z. Naturforsch. Sect. C Biosci.* **45**, 1–8

Schröder, J., Heller, W., and Hahlbrock, K. (1979a) *Plant Sci. Lett.* **14**, 281–286

Schröder, J., Kreuzaler, F., Schäfer, E., and Hahlbrock, K. (1979b) *J. Biol. Chem.* **254**, 57–65

Schüz, R., Heller, W., and Hahlbrock, K. (1983) *J. Biol. Chem.* **258**, 6730–6734

Schweizer, E., Müller, G., Roberts, L. M., Schweizer, M., Rösch, J., Wiesner, P., Beck, J., Stratmann, D., and Zauner, I. (1987) *Fat Sci. Technol.* **89**, 570–577

Schweizer, M., Takabayashi, K., Laux, T., Beck, K. F., and Schreglmann, R. (1988) *Nucleic Acids Res.* **17**, 567–586

Sherman, D. H., Malpartida, F., Bibb, M. J., Kieser, H. M., Bibb, M. J., and Hopwood, D. A. (1989) *EMBO J.* **8**, 2712–2725

Strebel, K., Beck, E., Strohmaier, K., and Schaller, H. (1986) *J. Virol.* **57**, 983–991

Vieira, J., and Messing, J. (1987) *Methods Enzymol.* **153**, 3–11

Wakil, S. J. (1989) *Biochemistry* **28**, 4523–4530

Wiesner, P., Beck, J., Beck, K.-F., Ripka, S., Müller, G., Lücke, S., and Schweizer, E. (1988) *Eur. J. Biochem.* **177**, 69–79

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene (Amst.)* **33**, 103–119

Zagursky, R. J., and Berman, M. L. (1984) *Gene (Amst.)* **27**, 183–191